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High-level expression of Na^+/D-glucose cotransporter (SGLT1) in a stably transfected Chinese hamster ovary cell line

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Abstract

The coding region of the high affinity Na^+/D-glucose cotransporter (SGLT1) was inserted into the eukaryotic expression vector GFP-N1 under the control of a CMV promoter. The plasmid was then stably transfected into a Chinese hamster ovary cell line (CHO). Transcription and synthesis of SGLT1 were proved by Northern and Western blot analyses. Transport activities of the transfected cells (G6D3) were examined by measuring the sodium-dependent uptake of α-methyl [^{14}C]D-glucoside (AMG). Kinetic analysis revealed a V_{max} of 10.3 nmol/min/mg (total cell protein) and a K_m of 0.26 ± 0.09 mM, respectively. The concentration of phlorizin required to inhibit AMG uptake by 50% in the presence of 0.1 mM AMG was 2.35 ± 1.84 μM. Electrophysiological studies showed that AMG induces a significant depolarization of membrane voltage in stably transfected CHO cells, suggesting an electrogenic Na-AMG symport. Immunoprecipitation with an antipeptide antibody yielded a nearly homogeneous polypeptide with a molecular mass of about 72 kDa. The amount of SGLT1 present in the CHO cell plasma membranes represents at least 1% of membrane protein, which is about 30–100 times higher than in natural sources, such as renal brush border membranes. In conclusion, the stably transfected G6D3 cells with a markedly high SGLT1 expression can serve as a promising model for studying cellular events related to Na^+/D-glucose cotransport and for analyzing the structure and function of the cotransporter itself.

Keywords: Na^+/D-Glucose cotransport; SGLT1; Transfection; Expression CHO cells; Immunoprecipitation; Diabetes

Abbreviations: CMV, cytomegalovirus; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylene glycolbis(oxyethylenenitrilo)]tetraacetic acid; GFP, green fluorescence protein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; KRH, Krebs-Ringer-HEPES; MEM, minimum essential medium; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; SSPE, saline sodium phosphate-EDTA; TX-100, Triton X-100

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1. Introduction

Na^+/D-Glucose cotransport could be demonstrated directly for the first time in isolated brush border membrane vesicles from kidney cortex and intestine two decades ago [1–6]. Two types of the Na^+/D-glucose cotransporter, SGLT1 and SGLT2, have been shown to exist in the kidney. The SGLT1 has a high affinity for D-glucose with a stoichiometry Na^+:D-glucose of 2:1, the SGLT2 has a low affinity with a stoichiometry of 1:1 [7,8]. Cloning expression provided detailed information about the primary sequences of SGLT1 and SGLT2. Functional expression of SGLT1 has been described in *Xenopus laevis* oocytes [9], COS-7 cells [10], and Sf9 cells [11]. Although attempts to purify and characterize these cotransporters using various biochemical approaches were undertaken by several investigators [12–17], thus far only partial purification has been achieved. This is mainly due to the low abundance of the cotransporter in the brush border membranes [18–20] and in the expression systems as well as to the lack of effective tools to specifically enrich the transport protein.

In this paper we, therefore, attempted to establish a high level expression system for SGLT1 and to apply immunological purification techniques. The level of expression observed in the stably transfected CHO cells was significantly higher than observed before. The expressed SGLT1 could be identified and immunoprecipitated with an antipeptide antibody 'Pan-3' raised against an amino acid sequence near to the C terminus. Our results suggest that the transfected cells represent a promising starting material for purification of the SGLT1 protein and may also serve as a potential model for the cellular events associated with an increased cellular uptake of D-glucose – a typical feature of diabetes.

2. Materials and methods

2.1. Construction of a SGLT1 plasmid

GFP and GFP fusion proteins have been used for different purposes in several mammalian cell lines [21–23]. The vector, pGFP-N1, used for cloning contains a strong promoter p_{CMV} and a multiple cloning site. The authors, therefore, felt that this vector would be suitable for the expression of SGLT1. First, the 5′ and 3′ non-translated regions of SGLT1 cDNA in pBluecript KS+ plasmid were removed by partial digestion with restriction endonucleases *Nco*I and *Eag*I. The 2000 bp fragment of SGLT1 cDNA [24] was then cloned into pET-28a, digested with the same enzymes, resulting in pET-sglt1. This plasmid was then digested with *Xho*I, filled with C and T and PolkI, and partially digested with *Nco*I. The resulting 2000 bp fragment was ligated with a 4000 bp fragment of pGFPN1 (to substantiate the origin of GFP gene), digested with *Bcl*I, filled with A and G and PolkI, and partially digested with *Nco*I. The resulting final plasmid pSGLT-5 was used for transfection experiments. A schematic presentation of this strategy is shown in Fig. 1.

2.2. Cell culture and stable transfection

CHO cells were subcultured in a six-well culture plate to 70% confluence in Dulbecco's modified Ea-

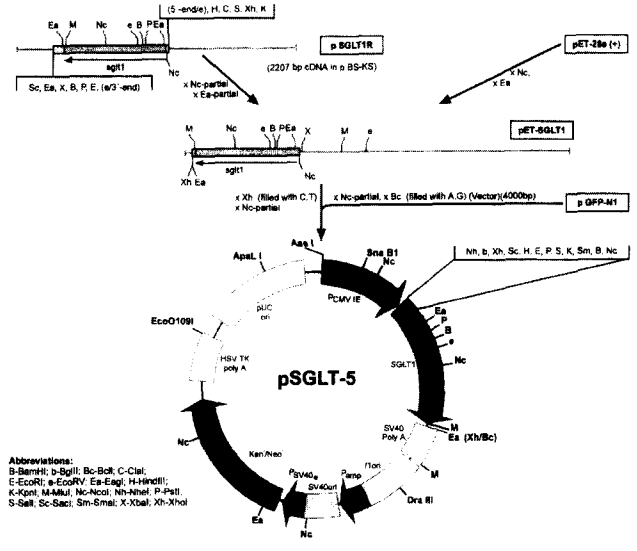

Fig. 1. Construction of pSGLT-5 using the eukaryotic expression vector GFP-N1. 5′ and 3′ non-translated regions of pBluescript KS+ plasmid containing 2207 bp of SGLT1 were removed by partial digestion with *Nco*I and *Eag*I. The 2000 bp fragment was cloned into pET-28a digested with the same enzymes. The resulting plasmid pET-SGLT1 was then digested with *Xho*I, filled with C and T and PolkI and partially digested with *Nco*I. The final fragment of 2000 bp was then ligated with the 4000 bp fragment of pGFP N1 previously digested with *Bcl*I, filled with A and G and PolkI and partially digested with *Nco*I.

gle's medium (DMEM), containing high glucose (25 mM) supplemented with 5% fetal calf serum, 1× minimal essential medium (MEM), 1 mM Na pyruvate, 25 μM β-mercaptoethanol, and 2 mM L-glutamine under 7.5% CO_2. Transfection of cells was carried out with a positively charged lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP; Boehringer Mannheim, Germany). In brief, 5 μg plasmid in 50 μl of 20 mM HEPES-Tris buffer, pH 7.4, were mixed gently with 100 μl of liposome suspensions (30 μg) in the same buffer in a polystyrene tube. The mixture was then added slowly to the cells (1×10^5/well) and covered with 5 ml of culture medium. The transfection was allowed to proceed for 20 h. A post-transfection period of 48 h after replacement with liposome-free medium followed. Subsequently, the cells were treated with trypsin (0.1%) and suspended in 1 ml fresh medium. The cell suspension was then diluted with 15 ml of medium containing geneticin G418 (200 μg/ml) in a plastic culture dish (diameter 10 cm) for selection and passages. Culture medium was renewed every second day and the culture continued for 30 days. For selection of pure SGLT1-positive clones, further selection and subculture were done by repeated dilution of the cell suspension (after treatment with trypsin) in a 96-well plate to achieve a state of one cell per well. Cells in wells were then grown in the same medium but with a higher geneticin concentration (400 μg/ml). In these wells also Na^+-dependent AMG uptake of cells was tested. Cells that showed the highest transport activity were selected for further growth and passages. Transfected cells multiplied from these wells were coded thereafter as G6D3.

2.3. Isolation of RNA and Northern blot hybridization

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Ten micrograms of RNA were subjected to electrophoresis on a 1.2% agarose gel in the presence of 16% formaldehyde. After electrophoresis, separated RNAs were transferred to a Nybond N membrane (Amersham Buchler, Braunschweig, Germany) according to the procedures described by Brown and Mackey [25]. The blot was hybridized with the randomly labeled 1200 bp *Nco*I fragment at 42°C for 16 h in a hybridization medium (50% formamide, 5×SSPE, 5×Denhardt's solution, 0.5% SDS, 100 μg/ml freshly denatured salmon sperm). As a control, the same RNA blot was hybridized with a RT-PCR amplified fragment of the β-actin gene (700 bp) under identical conditions. The RT-PCR was started with 0.3 μg of the total RNA isolated from the control and transfected CHO cells using the actin primers: 5′-ACCTTCAACACCC-C(A/C)GCCATGTACG-3′ and 5′-CT(A/G)ATC-CACATCTGCTGGAAGGTGG-3′.

After hybridization the membrane filter was washed with 2×SSC and 0.1% SDS for 5 min, 0.5×SSC and 0.1% SDS for 15 min, and 0.1×SSC and 0.1% SDS for 15 min at room temperature. The blot was washed twice with 0.1×SSC and 0.1% SDS at 65°C for 20 min and subjected to autoradiography on a Hyperfilm (Amersham Buchler).

2.4. Production and characterization of the antipeptide antibody (Pan-3)

A polyclonal antipeptide antibody, Pan-3, was raised against a deduced sequence, A606, near to the C terminus. The peptide A606 (LFCGLD-QDKGPKMTKEEEAAMKLKLC) was synthesized in our institute using a solid-phase peptide synthesizer (model 431A; Applied Biosystems). The purity of the peptide was examined on HPLC. Pan-3 was commercially produced (Cambridge Research Chemicals, Northwich, UK) by immunizing sheep with the peptide keyhole limpet hemocyanin conjugate. The antibody was then immunopurified through an affinity column conjugated with the respective peptide.

To characterize the antibody membranes were prepared from rabbit kidney, rabbit intestine (duodenum and jejunum), rat kidney, rat hepatocytes, and pig kidney. Membranes were solubilized in 1% TX-100 and 20 μg subjected to electrophoresis and Western blotting as described below. Anti-sheep IgG peroxidase (Sigma, St. Louis, MO, USA) was used to visualize the SGLT1-antibody complex.

2.5. Immunochemical detection of SGLT1-related proteins

Cells from two wells were washed three times with KRH-Na (Krebs-Ringer-HEPES; 120 mM NaCl, 5.6

mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 20 mM HEPES-Tris, pH 7.5) medium at 37°C and rinsed once with an ice-cold hypotonic buffer (10 mM HEPES-Tris, 5 mM EGTA, pH 7.4). Two to 3 ml of ice-cold hypotonic buffer containing protease inhibitors (aprotinin and leupeptin, each 20 μg/ml) were then added to the wells to lyse the cells at 4°C. Cells were directly removed from the wells with the aid of a rubber policeman, without a trypsin treatment and collected in a tube. Cell organelles were spun down at $100\,000 \times g$ for 60 min to exclude cytosolic proteins. The pellets were solubilized with 1% TX-100 (200 μl) in the same buffer at 4°C for 30 min. The non-solubilized material was spun down at $100\,000 \times g$ for 1 h in an airfuge (Beckman Instruments, Munich, Germany) at 4°C. The clear supernatant was stored at −70°C or immediately denatured under reducing conditions (2% SDS, 5% mercaptoethanol, 10 mM Tris-HCl, pH 7.5) for gel electrophoresis. Electrophoresis was carried out on a pre-cast SDS Tris-glycine gradient polyacrylamide gel (8–16%; Novex, San Diego, CA, USA). Routinely, 10–15 μl were applied to each lane. Separated polypeptides were electrophoretically blotted to a nitrocellulose or a polyvinylidene difluoride membrane. The blot was then blocked with 5% fat-free milk solution and incubated with a polyclonal antipeptide antibody Pan-3. In some experiments, the incubation was performed simultaneously in the presence of excess molar amounts of the corresponding antigen peptide, A606. Procedures for detection of Western blots using the enhanced chemiluminescence (ECL) system were used according to those described in the manufacturer's protocol (Amersham Buchler).

2.6. Transport studies

Na^+/D-Glucose cotransport activity of the G6D3 cells was determined by means of α-methylglucoside (AMG) uptake. CHO cells (non-transfected) and G6D3 cells were cultured in DMEM in a six-well plastic culture plate to confluence. Before the transport assay cells were incubated in a D-glucose-free culture medium for 1–2 h at 37°C, with one exchange of the medium. This maneuver was designed to lower the intracellular D-glucose level to a negligible amount so that the uptake of AMG would not be significantly affected. Cells were then washed twice with an osmotically balanced medium KRH-Na or KRH-choline at 37°C. One milliliter of 0.1 mM of AMG (containing 0.5 μCi of ^{14}C-labeled AMG) in KRH-Na or KRH-choline was added to the wells. After incubation for 15 min at 37°C the uptake of AMG by cells was terminated by removing the medium and rinsing the cells quickly with ice-cold KRH-Na or KRH-choline, containing 0.5 mM phlorizin. Cells were solubilized in 1.5 ml of 2% SDS containing 2 mM EDTA under vigorous agitation for 30 min at 25°C. Aliquots (400 μl) were taken for scintillation counting of radioactive AMG and for protein determination according to the method described by Lowry [26].

To examine the inhibition of the Na^+/D-glucose cotransport activity by phlorizin [20,27–29], [^{14}C]AMG uptake was carried out in the presence of sodium or choline as described above and in the presence of various concentrations of phlorizin ranging from 0.2 to 200 μM.

2.7. Electrophysiology

For the intracellular recordings, cells were grown on collagen-coated gas-permeable membranes in Petriperm culture dishes. In two pilot experiments, we found phlorizin-dependent AMG uptake rates of 10.1 and 8.2 nmol/mg cell protein/min under these conditions. This is virtually identical to the transport rates found for cells grown on six-well culture plates (see Section 3). Sheets of approx. 1 cm^2 covered with confluent monolayers were cut and transferred to the chamber. The cells were continuously superfused at a rate of 5 ml/min; changes of experimental solutions were complete within 15 s. All storage vessels, the superfusion lines, and the chamber were maintained at 37 ± 0.5°C. Two-channel microelectrodes were pulled from 1.5 mm o.d. 'Thick Septum Theta' glass capillaries (WPI, New Haven, CT, USA) on a Kopf vertical puller (750; David Kopf Instruments, Tujunga, CA, USA). One channel was used to measure voltage, the second to inject constant current pulses. The channels were filled with 0.5 mol/l KCl and had resistances of 150–200 MΩ with the electrode immersed in control solution. An Ag-AgCl wire in series with an agar-KCl (0.5 mol/l) bridge was used as the reference electrode. The experimental chamber

was mounted on the stage of an inverted microscope (IM35; Zeiss, Oberkochen, Germany). Cell impalements were done from above at an angle of 45° on a custom-made vibration-damped table under 320× magnification using a piezo-manipulator (PM 500-20; Frankenberger, Germering, Germany). Cell membrane voltages and input resistances were determined by means of a high-impedance electrometer combined with a current-injection unit (Frankenberger). Only negative current pulses were applied to avoid activation of voltage-dependent Na^+, Ca^{2+}, and K^+ conductances [30]. Pulse amplitude and duration were 10 pA and 1 s, respectively, and pulses were applied every 10 s. Criteria for successful impalements were as follows. (1) An abrupt change in voltage of some −15 to −20 mV upon impalement, followed by a period of approx. 1–2 min before stable recordings were obtained; this initial phase was always accompanied by a significant increase in input resistance and, thus, is likely to reflect sealing of the membrane around the electrode tip. (2) Stable potentials within ±2 mV in control solution. (3) A return of measured voltages to 0±2 mV upon withdrawal of the microelectrode.

2.8. *Immunoprecipitation of the SGLT1-related polypeptide*

Solubilized proteins obtained from one six-well culture plate (approx. 1.2 mg protein/well) were incubated with 4.5 μl of Pan-3 (0.8 mg/ml IgG) and 200 μl (50% suspension) anti-sheep IgG-Sepharose gel (binding capacity: approx. 1–2 μg Pan-3/100 μl gel) for 1.5 h at room temperature or overnight at 4°C. The unbound protein was removed by centrifugation. The gel was washed three times with a buffer containing 500 mM NaCl and 0.05% Tween 20. The gel was then further washed with 10 mM HEPES-Tris (pH 7.5) containing 0.05% Tween 20 to remove salt from the gel. The gel was suspended in 300 μl of the same buffer. Finally, the Na^+/glucose cotransporter-related polypeptide bound to the antibody complex was eluted by addition of 60 μl peptide antigen A606 (0.125 mg/ml) to the gel suspension. After incubation for 1 h at room temperature with gentle rotation the solution was separated from the Sepharose gel by centrifugation through a microfiltration tube with a built-in 0.22 nm membrane filter.

2.9. *[^{35}S]Methionine labeling and biotinylation of cell surface proteins*

G6D3 and CHO cells were grown in DMEM medium in six-well culture plates to approx. 80% confluence. After washing with a methionine-free medium, the cells were further cultured in the same medium containing 0.17 mM methionine and 88 μCi/well of [^{35}S]methionine (spec. radioactivity: 1416 Ci/mmol; ICN Biomedicals, Eschwege, Germany). The culture was continued for a further 16 h until harvest. For biotinylation of cells the method described by Collier et al. [31] was followed. Briefly, cells in a culture plate were washed twice in an amphibian Ringer solution (in mM: 110 NaCl, 4.0 KCl, 2.5 $NaHCO_3$, 1.0 K_2HPO_4, 2.0 $CaCl_2$ and 11 glucose, pH 7.8) at 4°C. Stock solution of sulfo-NHS-biotin in DMSO (200 mg/ml) was then added to the wells to result in a final biotin concentration of 0.5 mg/ml. The cells in wells were kept on ice for 20 min. Biotinylation was terminated by removing the medium and adding biotin-free medium containing 20 mM Tris-HCl or glycine (pH 7.5).

For measurements of incorporated [^{35}S]methionine and/or biotin-labeled surface proteins cells were washed twice with KRH-Na and 'total cell protein' was extracted by solubilization in 1% TX-100 in a Tris-HCl buffer (10 mM), including 5 mM EGTA and protease inhibitors. Supernatant obtained after centrifugation at 100 000×g for 1 h was divided into several aliquots for further analysis. Samples of 10 μl were taken for counting the total ^{35}S labeling and for trichloroacetic acid (TCA) precipitation to determine the TCA precipitable proteins. To assure a complete precipitation with TCA, a small amount of sodium deoxycholate was present in the TCA solution as described by Peterson [32]. One hundred microliters of the sample were used for immunoprecipitation with the IgG-Sepharose/Pan-3 complex as described above and another 100 μl were used for precipitation with avidin-Sepharose. Fifty microliters of avidin-Sepharose beads (50% slurry) were resuspended in 400 μl TBST-200 including protease inhibitors. One hundred microliters of solubilized ^{35}S-labeled CHO or G6D3 proteins were then added to the bead suspension and rotated slowly for 2 h at room temperature. The beads were separated from the suspension by centrifugation and washed twice with a 20 mM

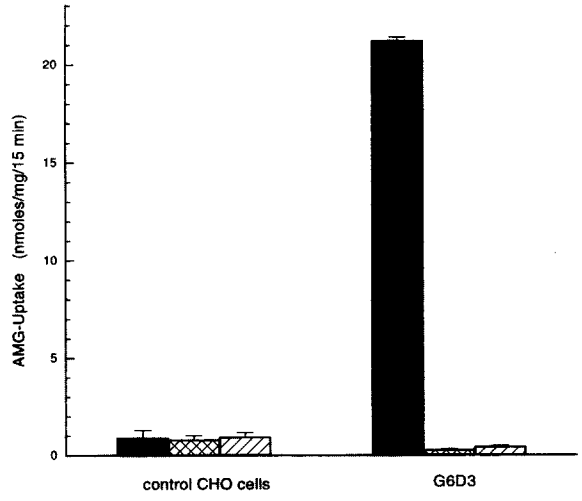

Fig. 2. [^{14}C]AMG uptake by non-transfected and stably transfected CHO cells G6D3. Cells were D-glucose depleted prior to transport experiments, as described in Section 2. Uptakes were measured by incubation with 0.1 mM [^{14}C]AMG for 15 min at 37°C in the presence of NaCl (black bar), or NaCl plus 0.5 mM phlorizin (cross-hatched bar), or in the presence of choline (hatched bar). One representative experiment is shown (means ± S.D. from three wells).

HEPES-Tris buffer to remove salts. The proteins bound to the beads were then directly denatured with 2% SDS. After centrifugation the clear solution was divided into several portions for counting of ^{35}S labeling and electrophoresis.

2.10. Materials

The eukaryotic expression vector pGFPN1 was purchased from Clontech (Palo Alto, CA, USA), pBluescript KS+ plasmid from Stratagene (La Jolla, CA, USA), pET-28a from Novagen (Madison, WI, USA), restriction enzymes from New England Biolabs (Beverly, MA, USA), and RNeasy kit from Qiagen. CHO cells were from the American Type Culture Collection (Rockville, MD, USA). Tissue culture media and G418 were from Gibco BRL (Eggenstein, Germany). Lipid (DOTAP) was purchased from Boehringer Mannheim. α-Methyl[^{14}C]D-glucopyranoside (spec. radioactivity 250 mCi/mmol) was from DuPont NEN (Bad Homburg, Germany). ECL reagents and Hyperfilm were from Amersham. Anti-sheep IgG-HRP (horseradish peroxidase) was from Sigma. Pre-cast SDS-polyacrylamide gradient gels (8–16%) were obtained from Novex (San Diego, CA, USA). All other reagents were of the highest grade commercially available.

2.11. Statistics

Results are expressed as mean values ± S.E.M. Student's *t*-test was employed for evaluation of differences; a difference was considered significant at $P < 0.05$.

3. Results

3.1. Functional expression of α-methyl-D-glucoside transport

The Na^+/D-glucose transport activities of CHO cells, in the presence of either NaCl or choline chloride, were used as selection criterion. As shown in Fig. 2, in non-transfected CHO cells no sodium-dependent or phlorizin-inhibitable AMG uptake could be detected. In contrast, AMG uptake by the transfected G6D3 cells was 22.9 ± 7.5 nmoles/mg cell protein/15 min (mean ± S.D.; $n = 10$) in the presence of NaCl and 0.35 ± 0.16 nmoles/mg cell protein/15 min (mean ± S.D.; $n = 10$) when 0.5 mM phlorizin was present. In the choline-containing medium, the uptake was as low as the basal values. Expression of

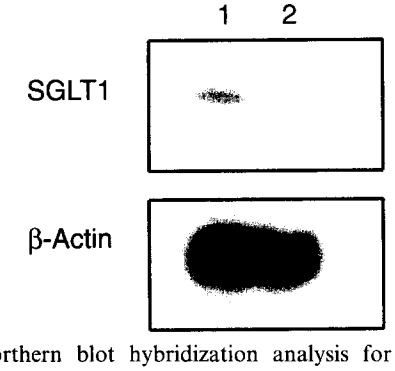

Fig. 3. Northern blot hybridization analysis for detection of SGLT1 expression at the mRNA level. Ten micrograms of total RNA isolated from freshly harvested G6D3 cells were applied to electrophoresis on formaldehyde-agarose (16% formaldehyde) gel. The separated poly(A)$^+$mRNA was then hybridized as described in Section 2. Upper panel: hybridization with a *sglt1* probe. Lane 1, mRNA isolated from the stably transfected G6D3 cell line; lane 2, RNA isolated from non-transfected CHO cells. Lower panel: hybridization with a β-actin DNA probe.

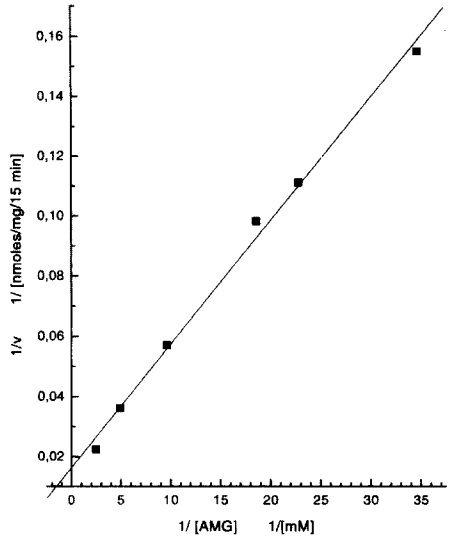

Fig. 4. Kinetics of sodium-dependent AMG accumulation in the G6D3 cells. Uptakes were measured at various AMG concentrations, ranging from 0.025 to 0.4 mM in the presence of sodium or choline in the transport medium. One representative experiment is shown. Each point shows average values from cells in two different wells.

SGLT1 in transfected CHO cells and its absence in non-transfected cells was confirmed by Northern blots (see Fig. 3). A positive signal for SGLT1 mRNA can only be seen in the transfected G6D3 cells. In contrast, expression of β-actin is observed both in control and in transfected cells. The sodium-dependent uptake showed an apparent K_m for AMG of 0.26 ± 0.09 mM (mean ± S.E.; $n = 3$) and a V_{max} of 10.3 ± 3.1 nmoles/min/mg cell protein (mean ± S.E.; $n = 3$), respectively (Fig. 4). The inhibitory efficiency of phlorizin on AMG uptake is shown in Fig. 5. A sigmoidal curve was obtained and a 50% inhibition of the AMG uptake (at 0.1 mM AMG concentration) was found at 2.4 ± 1.9 μM (mean ± S.E.; $n = 3$). An attempt to determine sodium-dependent [^{3}H]phlorizin binding in cell monolayers failed at 1 μM and 5 μM phlorizin since the difference of [^{3}H]phlorizin measured in the NaCl medium and in the choline medium was too small.

3.2. Electrophysiological evidence for Na^+/AMG cotransport

Activity of the Na^+/glucose cotransport system was also studied by electrophysiological techniques. In intracellular recordings with conventional microelectrodes, 1 mmol/l AMG depolarized membrane voltage from −26.8 ± 2.3 to −15.5 ± 1.0 mV, i.e. by 11.3 ± 2.5 mV ($n = 6$; $P < 0.01$) with the maximal effect occurring at 29 ± 4 s after change of the superfusate (Fig. 6). In parallel, cell input resistance decreased from 1.04 ± 0.18 to 0.87 ± 0.17 GΩ, i.e. to 82.6 ± 3.0% of the control ($P < 0.01$). In the continuous presence of AMG, membrane depolarization then declined to 74.4 ± 5.2% within 5 min. Upon removal of the sugar, both membrane voltage and input resistance slowly returned to control values. These data clearly demonstrate the presence of electrogenic AMG uptake in the transfected cells.

3.3. Identification of SGLT1-related proteins

To further characterize the expressed SGLT1, Western blot analysis was performed; thereby the antipeptide antibody Pan-3 raised against the sequences close to the C terminus of SGLT1 and shown to recognize the cotransporter-related poly-

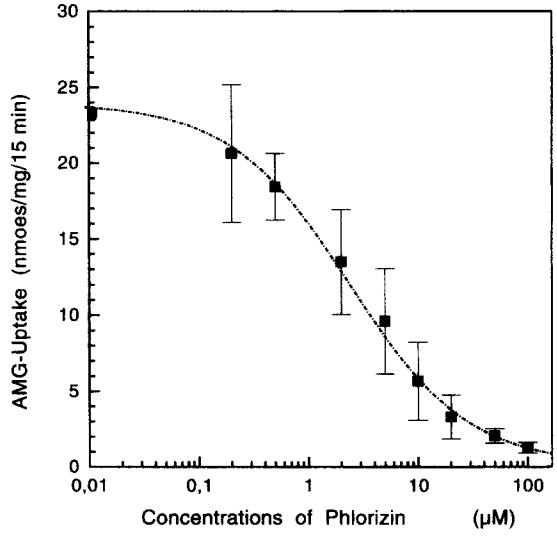

Fig. 5. Inhibition of AMG uptake of G6D3 cells as a function of phlorizin concentrations. Immediately after depletion of D-glucose cells were washed three times with KRH-Na buffer and incubated with the same buffer including in addition 0.1 mM [^{14}C]AMG and phlorizin of varied concentrations, as indicated (0.2–200 μM) for 30 min at 37°C. The incubation media were removed, the cells quickly washed three times with ice-cold AMG- and phlorizin-free buffer and solubilized. The cells were then solubilized. Aliquots were subjected to counting of radioactivity and protein determination. For each phlorizin concentration uptakes from three wells were collected. Data represent means ± S.E. ($n = 3$).

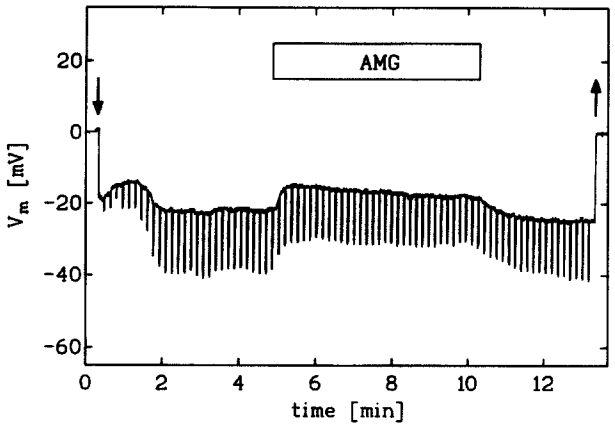

Fig. 6. Effects of 1 mmol/l AMG on membrane voltage (V_m) and input resistance in transfected CHO cells. A cell in a confluent monolayer was impaled at ↓ and the electrode was withdrawn at ↑. Vertical deflections result from injected current pulses of 10 pA. Note the pronounced depolarization of V_m and the coinciding decrease in input resistance (voltage deflections) upon addition of AMG to the superfusate.

peptide in several tissues was used (see Fig. 7). Pan-3 recognized the polypeptide in the brush border membranes from rabbit, rat and pig kidney cortex and in the rabbit intestinal brush borders at the same position (72 kDa) as an intense and broad signal. By contrast no reaction was observed in membranes of hepatocytes, where no SGLT1 is present. As shown in Fig. 8, Pan-3 recognizes a strong polypeptide band at 72 kDa in the organelle extract of the transfected cells (lane A). This antigen antibody reaction was suppressed upon addition of the peptide A606 to the incubation mixture (right panel of Fig. 8). The size of this band was identical to that detected in brush border membranes (lane C) isolated from the cortex of rabbit kidney. Two additional bands at a higher molecular size were also visible. Both were also absent in the presence of the peptide. These bands could possibly be oligomers of SGLT1 as reported earlier [13]. No detectable bands were seen in the extract from non-transfected CHO cells (lane B).

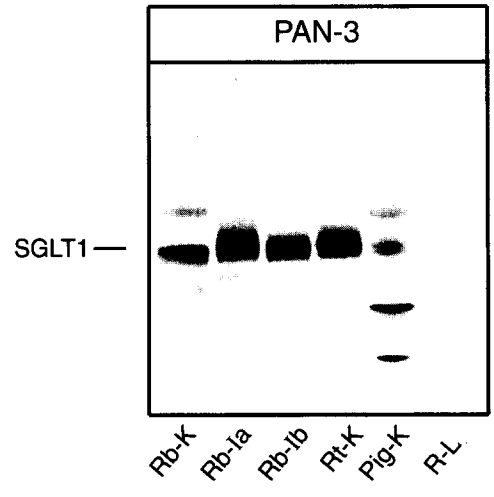

Fig. 7. Immunological characterization of polyclonal antipeptide antibody, Pan-3. Membranes were prepared from rabbit kidney (Rb-K), rabbit intestine (duodenum (Rb-Ia) and jejunum (Rb-Ib)), rat kidney (Rt-K), pig kidney (Pig-K), and rat liver (R-L). The membranes were solubilized and subjected to a Western blot analysis (see Section 2) in the presence and absence of the peptide antigen A606. In the presence of A606 the signals detected with Pan-3 are suppressed (data not shown).

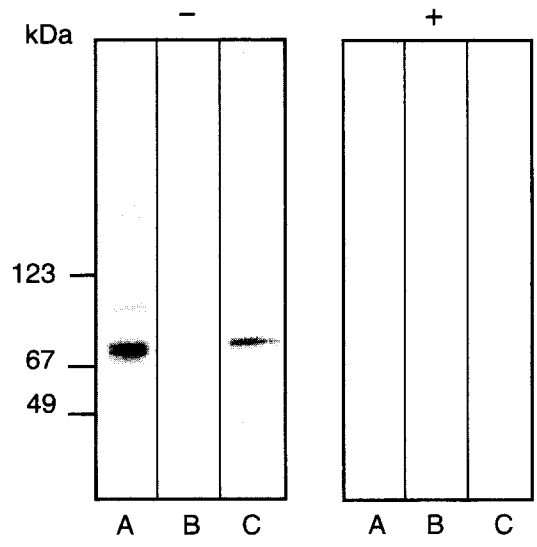

Fig. 8. Western blot analysis to show the expression of SGLT1. Solubilized cell organelle proteins (see Section 2) of stably transfected CHO cells G6D3 (A), non-transfected CHO cells (B), and brush border membranes from rabbit kidney cortex (C) (20 μg each) were subjected to electrophoresis on a SDS-polyacrylamide gradient gel (8–16%) with subsequent electrotransfer to a nitrocellulose membrane. The presence of SGLT1-related polypeptide was detected using the immunopurified polyclonal antipeptide antibody, Pan-3 (0.8 mg/ml) at a dilution of 1000-fold. The pattern on the right side panel (denoted +) was obtained under identical conditions except that the peptide A606 (0.125 μg/ml) was simultaneously present with the antibody Pan-3 during incubation.

3.4. *Estimation of the amount of SGLT1 proteins in the stably transfected cell line*

Since a direct measurement of [^{3}H]phlorizin bind-

Table 1
(a) Estimation of the amount of plasma membrane protein and SGLT1 protein in transfected CHO cells by methionine and biotin labeling

		Cell protein/dish (mg)	^{35}S in TCA fraction of total cell protein (cpm $\times 10^7$/mg cell protein)	^{35}S in immunopurified fraction (cpm $\times 10^4$/mg)	Avidin bound (cpm $\times 10^6$/mg total label)[a]	Amount of ^{35}S present in immunopurified fraction (% of protein)	Surface protein bound to avidin (% of total cell protein)
Non-biotinylation	G6D3	0.45	8.0	9.7	–	0.05	–
Biotinylation	CHO	0.44			3.32	–	5.3
	G6D3	0.29	8.0	9.5	3.33	–	4.1

CHO and G6D3 cells were grown in plastic dishes (10 cm diameter). [^{35}S]Methionine was added to the cells as described in Section 2. One dish from each cell type was used for biotinylation. The cells were collected and solubilized with 1% for TX-100 in the presence of protease inhibitors and 5 mM EGTA (see Section 2) at 4°C and centrifuged at $10000 \times g$ for 30 min in the cold. The supernatant was then divided into aliquots of different sizes and subjected to protein determination, ^{35}S incorporation, immunoprecipitation or avidin binding. The cpm values obtained were the averages of three measurements. [a]Values for transfected cells were corrected where applicable for the results obtained with non-transfected CHO cells.

(b) Estimation of the amount of SGLT1 expressed in transfected CHO cells using immunoprecipitation and protein determination

	Exp. I	Exp. II	Exp. III
Total cell protein	370 mg	545 mg	255 mg
Solubilized protein	135 mg	185 mg	110 mg
Immunopurified fraction	210 μg	300 μg	212 μg
% of immunopurified protein	0.056%	0.055%	0.083%

Confluent G6D3 cells in plastic dishes (10 cm diameter) were washed and harvested in a hypotonic buffer including 5 mM EGTA and protease inhibitors. Samples were taken for measuring the protein content. Cells were then collected in a tube and centrifuged at approx. $3000 \times g$ for 15 min. The cell pellets were incubated with the same buffer including 1% TX-100 followed by centrifugation at $100000 \times g$ for 1 h. Solubilized proteins (the supernatant fraction) were then diluted 5-fold with a Tris-HEPES buffer containing 0.05% Tween 20 and 200 mM NaCl (TBST-200). For further details of purification see Section 2.

ing was difficult, as mentioned above, two other approaches were used to estimate the amount of SGLT1 present in the plasma membrane. The first approach was the comparison of the amount of [^{35}S]methionine recovered in the surface membrane proteins of G6D3 cells with the amount found in the immunoprecipitate. First, all cell proteins were steady-state labeled with [^{35}S]methionine. Thereby it was assumed that [^{35}S]methionine was incorporated randomly into all proteins. Then cell surface proteins were biotinylated [31]. The results of such an experiment are listed in Table 1a. In case of non-transfected CHO cells biotinylated surface proteins that are bound to avidin-Sepharose were about 4–5% of the TCA-precipitated proteins. Virtually equal amounts of surface protein were biotinylated in G6D3 cells. The immunoprecipitated SGLT1 proteins accounted for about 0.05% of the TCA-precipitated proteins, representing about 1% of the surface membrane proteins.

The second approach was a direct measurement of the protein amount in the immunopurified fraction. As shown in Fig. 9A, the immunoprecipitated fraction is highly enriched in SGLT1. The immunoprecipitable SGLT1 protein in all three experiments lies between 0.05 and 0.08% of the TCA-precipitable proteins, in agreement with the finding obtained from the biotinylation experiment (see Table 1b).

In addition, in Fig. 9B the immunoreaction in the Western blot of the transfected CHO cell homogenate with that in a brush border membrane sample is compared. Quantitative analysis of ten samples revealed an enrichment of SGLT1 (as judged by immunoreactivity) of 2.55 ± 0.3 in the CHO cells compared to the brush border membranes. Assuming that about 5% of the total protein represents membrane protein, an enrichment of 50-fold in the CHO cell plasma membranes compared to the brush border membranes is achieved.

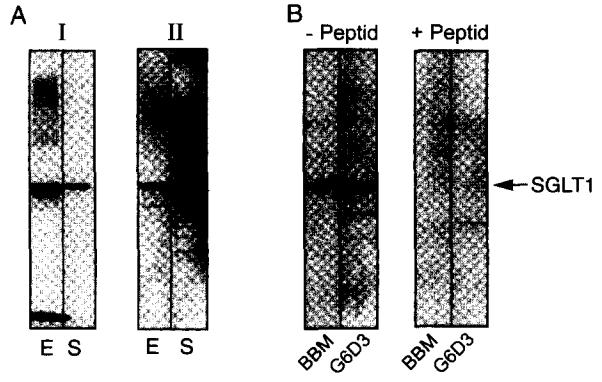

Fig. 9. (A) Immunoprecipitation of SGLT1 overexpressed in transfected CHO cells. I, Western blot; II, gold staining. E denotes the immunopurified protein fraction, S the proteins extracted from the cell organelles. For E 2.5 μg of proteins and for S 25 μg of proteins were applied. (B) Comparison of immunoreaction of Pan-3 in brush border membranes (BBM) and G6D3 cells. Twenty micrograms of solubilized proteins were applied for each lane. Western blot analysis was performed in the absence (−) and presence (+) of A606.

4. Discussion

In the current studies the established CHO cell line has been chosen as an expression system. Several reasons were considered advantageous compared with other known systems. (1) The cells are easy to maintain and remain stable and viable over prolonged periods of passages. Stably transfected cells can also maintain their genetic information after many passages. (2) A Na^+/D-glucose cotransport is not present in non-transfected cells. (3) Though not polarized, the plasma membrane of CHO cells might be structurally more comparable to mammalian membranes, where SGLT1 has been cloned from, than for instance the plasma membrane of *Xenopus* oocytes. (4) Transport studies can easily be conducted and experimental design can be altered to suit the need of investigations. (5) As tumor cells, CHO cells have a high glycolytic activity which should prevent an intracellular accumulation of D-glucose by the newly synthesized SGLT1. Such an accumulation could be theoretically more than 1000-fold, a dramatic challenge to the maintenance of cell volume. Indeed, measurements of intracellular D-glucose (unpublished results) revealed an amount of only 285 nmoles/mg of cell protein, which would translate into 95–28.5 mmoles/l, assuming an intracellular volume of 3–10 μl/mg of protein.

As shown in an overexpression of up to 49-fold of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in hepatoma cells of gluconeogenic rats [33], the plasmid construct containing the transcription units under control of the cytomegalovirus promoter and SV40 polyadenylation genome seems able to induce a high level expression. With a two-step strategy over the plasmid pET-28a(+) we were able to replace the open reading frame (716 bp) of GFP (green fluorescent protein) with a partially digested *sglt1* fragment (2000 bp). The resulting plasmid pGFP-SGLT1, containing the GFP vector pGFP-N1, was then stably transfected into CHO cells with success. For transfection, the positively charged liposomes of DOTAP were chosen because of low cytotoxicity that ensures a good viability of transfected cells.

The polyclonal antibody Pan-3 raised against the sequences close to the C terminus recognizes the SGLT1-related polypeptide close to 72 kDa in rabbit kidney cortex and in other tissues (Fig. 7). The same antibody also evidently reacts with a polypeptide of the same size only in G6D3 but not in control CHO cells. This finding, together with the functional tests and Northern blot analysis, demonstrates that the SGLT1 polypeptide is expressed after transfection. Electrophoretic analysis revealed additional broad bands at a higher molecular weight. The reaction of these bands with the antibody was also suppressible with the peptide. This finding could suggest the presence of aggregates that are stable enough even under relatively mild reducing conditions [34]. Whether these aggregates represent oligomers of the Na^+/D-glucose cotransporter that first have been postulated on the basis of radiation inactivation studies [13] and confirmed by several investigators [35,36] or unspecific complexes caused by overexpression remains to be elucidated.

The estimated K_m for AMG is 0.26 mM, which is very close to the high affinity transport reported in the medullary brush border membranes (BBMV) of rabbit kidney [8]. The half-concentration of phlorizin required to inhibit the sodium-dependent AMG uptake of 2.5 μM is very similar to the value found in LLC-PK_1 cells [37]. Taken together, the results from transport experiments obviously suggest a similar behavior of the expressed SGLT1 compared with the native system.

Comparing different expression systems based on uptake data is generally difficult. The V_{max} of 10 nmoles/mg cell protein/min observed in our studies is much higher than that found in other expression systems, such as *Xenopus* oocytes [9] and transfected COS-7 [10]. Since according to our results only 5% of the total cell protein represents cell surface proteins, the V_{max} per mg of membrane protein can be estimated to 200 mmoles/min, 50 times higher than the 4 nmoles/mg membrane protein/min determined in rabbit renal brush border [4,5,8]. A 50-fold higher content of SGLT1 in the CHO cell membranes can also be deduced from the labeling experiments and the protein determinations.

The microelectrode data of the present study are in good agreement with a patch-clamp analysis on CHO cells performed by Skryma et al. [30]. His group reported an average membrane voltage and a range of membrane resistance of -37 ± 11 mV and 800 MΩ–1 GΩ, respectively. Moreover, the high input resistances found in our study strongly argue against a significant electrical coupling of CHO cells in confluent monolayer. In other words, cell input resistance most probably equals cell membrane resistance that justified the use of the former parameter for a quantitative comparison of electrophysiological data with those derived from the AMG uptake. In the present study, CHO cells exhibit a phlorizin-inhibitable AMG uptake of 10.2 ± 2.3 nmol/mg protein/min. Because 1 mg protein equals approx. 10^6 cells, this value is equivalent to an uptake of 0.17×10^{-15} mol/cell/s. From this, and because the stoichiometry of SGLT1 is two Na^+ vs. one glucose [7,8], a Na^+ inward current of 0.17×10^{-15} mol/s $\times$ 96 500 C/mol $\times$ 2 = 32.8 pA can be calculated for a single cell. The average input resistance of CHO cells equalled 1.04 GΩ in the present study. By assuming that in a confluent monolayer approximately one third of the total cell surface may be accessible to rapid changes of the superfusate we would end with an expected AMG-induced membrane depolarization of 1/3 $\times$ 32.8 pA $\times$ 1.04 GΩ = 11.4 mV. This finding is very close to the value observed in our microelectrode experiments. The electrophysiological studies thus clearly provide evidence that AMG uptake in the presence of sodium chloride is electrogenic and proceeds via sodium/AMG symport. In addition, they confirm the results obtained in the flux studies.

In conclusion, our results suggest that the plasmid construct pSGLT-5, containing the pGFP-N1 vector and the promoter p_{CMV}, can induce a high level of SGLT1 expression in stably transfected CHO cells. The clone G6D3 possesses a great potential to be used as a model to study the intracellular mechanisms of biosynthesis of SGLT1 and to produce this protein in large quantities that could be sufficient for structural and functional analysis.

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